University of Alberta Library
0 1620 3416240 2

For Reference

NOT TO BE TAKEN FROM THIS ROOM

FACTORS INFLUENCING THE GROWTH CURVES OF RHIZOBIA IN HUMUS AND SOIL CULTURES.

Robert A. Hedlin, B. S. A.
University of Alberta.

April 1947.

Ex libris
Universitatis
Albertaensis

UNIVERSITY OF ALBERTA
QUAECUMQUE VERA

University of Alberta

Faculty of Agriculture

This is to certify that the undersigned have read and recommend to the Committee on Graduate Studies for acceptance, a thesis submitted by R. A. Hedlin, B. S. A., entitled, "Factors Influencing Growth Curves of Rhizobia in Humus and Soil Cultures".

J. D. Newton

C. F. Bentley

University of Alberta
Faculty of Agriculture

This is to certify that the undersigned have read and recommend to the Committee on Graduate Studies for acceptance, a thesis submitted by R. A. Hedlin, B. S. A., entitled, "Factors Influencing Growth Curves of Rhizobia in Humus and Soil Cultures".

Digitized by the Internet Archive
in 2018 with funding from
University of Alberta Libraries

https://archive.org/details/factorsinfluenci00hedl

Thesis
1947
#13

FACTORS INFLUENCING THE GROWTH CURVES OF RHIZOBIA IN HUMUS AND SOIL CULTURES.

Robert A. Hedlin, B. S. A.
Department of Soils.

A THESIS

submitted to the University of Alberta in partial
fulfilment of the requirements for the degree of

MASTER OF SCIENCE.

Under the direction of
Dr. J. D. Newton.

Edmonton, Alberta. April, 1947.

Thesis
1947
#13

TABLE OF CONTENTS.

Page

1. Introduction 1
2. Review of Literature
    (a) Method of Culturing Rhizobia and of Applying Inoculant to Seed........... 6
    (b) Factors Which Affect the Growth of Rhizobia in Soil and in Artificial Cultures 13
3. Outline of the Investigation................ 25
4. Results
    (a) Plate Counts of Rhizobia and Other Microorganisms....................... 30
    (b) Readily Available Nutrients in the Four Inoculants Used................. 50
5. Summary 59
6. Acknowledgments............................ 62
7. References

: : : : : : : :

FACTORS INFLUENCING GROWTH CURVES OF RHIZOBIA IN HUMUS AND SOIL CULTURES.

R. A. HEDLIN.

* * * * * * *

INTRODUCTION.

When Hellriegel and Willfarth discovered the symbiotic relationship between rhizobia and leguminous plants in 1888, they opened a whole new field for research. Their discovery was one of those of which Pasteur once said, "What really leads us forward is a few scientific discoveries and their application".

Once the prominent agricultural scientists of Europe and America had confirmed the findings of Hellriegel and Willfarth, they set about to study means of making practical applications of the new knowledge. The study of legume inoculation has continued since that time. It is interesting to note that legume inoculation, of a sort, had been practiced many centuries before. In the second century B. C., the great Roman agriculturalist Cato wrote, "Sow for feed for cattle, clover, vetch, fenugreek, field beans and pulse. Sow these crops a second and a third time." While recommending crop rotation to farmers of his day, he noted that better results were obtained by growing legumes on the same field for several years. What he was unquestionably observing, although unaware of it, was what is commonly referred to as gradual inoculation - the spread of rhizobia through a field by

such agents as wind and running water.

This is not the only ancient agricultural practice which was based largely on legume inoculation. The transfer of soil from one field to another in order to improve the soil productivity is an old custom handed down from ancient times. The general practice when using this technique was to apply at a rate of several tons to the acre, soil rich in humus. This practice had a triple purpose - the addition of plant food, the addition of bacteria, and improvement of the physical properties of the soil. Salfeld (as reviewed by Fred et al (14)), reported that in 1883, three years before the Hellriegel discovery, he had noted the value of top dressing land with soil from another field. The benefit was more apparent if the transfer soil was from a field where leguminous crops had been grown successfully.

The concentrated study of the problem of legume inoculation about the turn of the century led to the development of two methods of carrying this out. The first of these to come into general use was the soil transfer method - similar to that described above except that 100-500 pounds of transfer soil were used per acre. This smaller amount of soil was adequate to serve the single purpose of inoculation. This method was used within a year of the discovery of the symbiotic relationship between rhizobia and legumes.

There were certain difficulties with the soil transfer method which made it rather unsatisfactory. Firstly, the physiological efficiency of the bacteria is now recognized to be of prime importance. It is not enough that nodules be formed. They must benefit the plant as shown by increased growth and increased total nitrogen in the tissues. The soil transfer method is a reliable

way to secure nodules but there is no assurance that they will benefit the host plant. In the second place since they are not applied directly to the seeds, infection may take place slowly. Thirdly, the cost of transporting 100-500 pounds, or even 1,000 pounds of soil per acre, is almost prohibitive if the acreage is large and the distance which the soil must be transported is great. In the fourth place, in transferring soil there is considerable danger that insects and weed seeds, as well as bacterial and fungous diseases may be carried with the soil.

For these reasons early in the study of legume inoculation the use of pure cultures was attempted. Numerous patents for the preparation of pure cultures were taken out during the early part of the present century. Many of these failed to live up to the extravagant claims made for them and many were less successful than the soil transfer method had been. However, because of the ease with which they could be applied, the use of pure cultures soon completely displaced the soil transfer method.

At the present time nearly all inoculation of legumes is carried out by applying to the seed commercial preparations consisting of peat or other high humus mixtures to which rhizobia have been added. These humus cultures have replaced the older jelly cultures which first appeared on the market about 1900. In those days the farmers did not object to the messy jelly inoculants, because they were using formaldehyde and other seed treatments which were just as difficult to handle. However, when Ceresan and other dust treatments were introduced for smut control, farmers demanded a better legume inoculant - one in dust form. Commercial

producers of inoculants tried to supply a satisfactory culture in powder form with the result that "Dust Kote" and several other air dry dusts appeared on the market. These dry cultures, however, were entirely unsatisfactory because bacteria introduced into a dry medium perished very soon and the inoculation material was almost worthless by the time it was applied to the legume seed.

The humus cultures in common use today are prepared by commercial companies in the late winter or early spring and shipped to retailers for sale to farmers. The culture is usually mixed with water to form a suspension which is applied to the seed just before seeding. Sometimes, also, the culture is applied to the seed in dry form.

In the use of these cultures poor results are sometimes obtained. Preliminary unpublished investigations of some of these cultures have been made by the University of Alberta, Department of Soils. The results indicate that the poor inoculation obtained with many commercial inoculants was due to the tremendous growth of microorganisms, other than rhizobia, after packaging. Actinomycetes particularly, were frequently found. The contaminants, either by production of growth inhibiting substances or by competition for food materials, caused a rapid decline in the numbers of rhizobia in the cultures.

The purpose of the study reported here was to make a further investigation of the factors which affect the growth curve of rhizobia in humus cultures. The study is also concerned with the length of time which rhizobia cultures can normally

be expected to supply bacteria in numbers which will give adequate inoculation of legume seed.

REVIEW OF LITERATURE.

A great deal of work has been done on production of commercial inoculants for leguminous plants. The greatest volume of literature on the subject was published between 1910 and 1915. That literature and, in fact, all the literature prior to 1932, is reviewed by Fred et al (14) in Chapter XIII of their book entitled "Root Nodule Bacteria and Leguminous Plants". Research work concerned with inoculation has dealt with a number of problems, chief of which have been: (1) methods of culturing rhizobia and of applying the inoculant to the seed; (2) factors which affect the growth of rhizobia in soil and in artificial cultures. It is proposed to review the literature under these headings.

(1) Methods of Culturing Rhizobia and of Applying the Inoculant to the Seed.

The first method of inoculating legumes was the soil transfer method introduced by Salfeld (as reviewed by Fred et al (14)). Within a few years this method was replaced by varying techniques whereby the bacteria were applied directly to the seed. In one of these methods for example, soil known to contain rhizobia was mixed with the seed just before planting. A modification of this procedure was reported by the Illinois Farmers' Institute (27) in 1913. This modification consisted of moistening the seed with a strong glue or sugar solution before the soil containing nodule bacteria was mixed with the seed. This procedure was intended to increase the amount of soil which would cling to the seed. Arny and McGinnis (7) noted that one bushel of seed treated with glue

or sugar solution held 6 to 12 pounds of soil. However, with few exceptions, the degree of nodulation obtained using this method was unsatisfactory. Much better results were obtained from the use of an equal weight of seed and soil without glue. Ames and Casanova (6) also obtained good results from the use of equal weights of seed and soil.

In 1896 Nobbe and Hiltner presented their first paper (as reviewed by Fred et al (14)) relating to the use of pure cultures of rhizobia for the inoculation of legumes. The cultures of bacteria were applied directly to the legume seed or to a small amount of soil, which was distributed over the field to be sown. A few months prior to the publication of this paper they had patented in England and America a process covering the preparation of these cultures of rhizobia. They used the trade name "Nitragin" for their commercial product, which was distributed in small bottles in which the bacteria grew on the surface of a jelly medium. Nobbe and Hiltner recommended that a suspension of the legume bacteria be made up from this culture and applied either directly to the seed or to a small amount of soil which was spread over the field and mixed in to a depth of about three inches. They favored this second method.

Criticisms of "Nitragin" cultures were made by other workers. This led to several other patents being taken out for commercial cultures during the early part of the twentieth century. These patents included the Hartleb culture named after its patentee (22), the Nitro-culture introduced by Moore (36) and the Azotogen culture brought out five years later by Simon (according

to Fred et al (14)). The patent taken out by Hartleb was essentially a method for inoculating seeds. The seeds were first washed with water and then inoculated with a suspension of rhizobia bacteroids. His patent included a method for producing bacteroids in the absence of the host plant. Moore (35) attributed the partial failure of Nitragin cultures to the use of a nitrogen rich medium. On such a medium, he argued, the bacteria grew well but lost their power to fix nitrogen. He obtained a patent for growing the bacteria in a medium low in combined nitrogen. Absorbent cotton was saturated with a culture of the desired organism, which had been grown in a medium having a relatively low nitrogen content. The absorbent cotton was dried, wrapped in tinfoil and shipped to the farmers. Farmers dissolved the contents of this package in a nutrient solution which was incubated for 48 hours before being applied to the seed. This culture was even less successful than the "Nitragin" culture had been, the failure probably being due to the fact that the bacteria did not live well in the absorbent cotton.

The Azotogen culture introduced by Simon (as reviewed by Fred et al (14)), was the first attempt to use moist soil as a culture medium for commercial inoculants. This method was based on the use of a pure culture of the desired strain of rhizobia developed in sterile soil. Thus the use of moist soil cultures was introduced. This is the type of culture in common use today.

J. C. Temple (52) reported on sterilized soil as a medium for growing rhizobia. He compared the numbers of rhizobia per gram of soil with the numbers of rhizobia in a nutrient solution.

He found the latter very inferior as a medium in which to grow the organisms. In sterile soil the soybean organisms reached a maximum of 380 million per gram, whereas in the nutrient solution the maximum number obtained was 65 million per c.c. Similar results were obtained using legume bacteria from crimson clover and alfalfa. The soil used by Temple was low in fertility and he found that by addition of various organic compounds such as alfalfa meal he was able to increase the numbers of bacteria very markedly. In this work, however, counts were not continued beyond the 42nd day.

In 1931 Hofer (25) made a study of the numbers of rhizobia in humus cultures. He reported that the numbers were highly variable. After one week the count was usually higher than at the time of inoculation, but after 50 days it was lower. After one year the count of nodule bacteria was only one per cent of what it had been immediately after inoculation. In his report no data concerning the numbers are given, nor is there an accurate description of the techniques used.

In a more recent investigation, Spaulding (51) reports that on the basis of yield tests agar cultures of rhizobia are better for inoculating peas and soybeans than are humus cultures. He used two kinds of agar cultures and three kinds of humus cultures. He suggests that humus may not be as favorable a medium as agar for maintaining the nitrogen fixing efficiency of the bacteria.

Albrecht (3) carried out an experiment designed to compare the efficiency of two dry inoculants with agar and soil inoculants. One of the inoculants tested was an air dry, chamois-gray, pulverized material similar in appearance to dry soil. The

other culture was a black gray material of peaty nature. These inoculants carried instructions for mixing them with the seed. Both the seed and inoculant were left in the dry condition. Such dry inoculated seed was supposedly ready to be seeded. It was also supposedly suitable for long time storage without harmful effects on the rhizobia.

In his experiments Albrecht applied the dry culture to the seed in various ways. He mixed the dry culture with seed as directed, and he applied the dry culture to moistened seed. Water suspensions of the dry culture were applied to the seed, and the dry culture was sifted on to moistened seed, followed by a dusting of finely powdered limestone. He compared the nodulation obtained in such experiments with that obtained when commercial agar cultures were applied to the seed, and also when a soil-crushed-nodule suspension was applied to the seed. His results indicate that in none of the methods of using dry inoculants were the nodule numbers on the alfalfa roots increased over the check to the same extent as was true for the commercial agar culture or for the soil-crushed-nodule culture. He concludes that it is doubtful if these dry cultures can serve to increase nodule numbers significantly.

Fellers (13) carried out experiments in which he made tests of three types of commercial cultures - agar cultures, liquid cultures, and soil and muck cultures. In this work he counted the number of bacteria using the plate method and also carried out cultural experiments to test the ability of the cultures to cause nodule production.

Fellers notes that the standard of purity of the cultures was high. Few of those claimed to be pure contained contaminants. However, he expresses doubt as to whether a pure rhizobia culture

makes a better legume inoculant than an impure one, especially in soil or muck cultures. Fellers points out that although most of the cultures studied were agar cultures, the use of peat is rapidly coming to the fore and will probably entirely displace the agar and liquid cultures. He gives several reasons for expecting this to happen:

1. Soil or muck cultures possess greater viability than agar or liquid cultures.
2. More bacteria are usually present in acre size soil or muck cultures than in acre size agar or liquid cultures.
3. In soil or muck there is better aeration and the medium is a more natural one than agar or liquid cultures.
4. When humus or muck are used, many types of rhizobia may be added to one culture.
5. Sterilization of soil or peat is not always necessary or desirable since some organisms present in soil aid the development of rhizobia.
6. Heat, light and exposure do not affect soil and peat cultures as much as agar or liquid cultures. Even after soil or peat becomes dry, it will support the growth of many bacteria.
7. The ease of inoculating with soil cultures gives them preference over liquid cultures.

Fellers found that the number of bacteria per cubic centimeter varied greatly. In general those cultures with less than one million organisms per c.c. were not effective, although it was impossible to set definite limits. He found that cultures

which gave high plate counts were usually satisfactory in giving nodulation of legume plants.

A number of papers have been published dealing with the effect of numbers of bacteria in the inoculum, on the size and number of nodules produced and of the relationship between degree of nodulation and degree of benefit to the host plant. Nobbe and Hiltner (as reviewed by Fred et al (14)), report that they detected no appreciable advantage in nodulation upon increasing the number of bacteria to 25 times the usual application. Quite different results were reported by Perkins (40), Wilson (58), (59), (61), and Thornton (54). These investigators report that, within limits, the number of nodules is increased when the number of bacteria is increased. Perkins observed an increase in nodules when the number of cells per seed increased up to 50, but with still greater numbers there was no gain. Wilson obtained similar results. Thornton, on the contrary, noted with alfalfa in a field experiment an increase in number of nodules when the number of bacteria per seed was raised from 2,500 to 20,000 organisms. In a pot experiment with runner beans, the use of more than 1,280,000 organisms per pot (six seeds per pot) was still effective in increasing the number of nodules, though the increase was not in direct proportion to the number of cells. Fred et al (14) feel, however, that Thornton's results do not disprove that there is a limit to nodulation imposed by the plant itself.

(2) Factors Which Affect the Growth of Rhizobia in Soil and Artificial Cultures.

Fred et al (14) in discussing the factors which influence the growth of rhizobia stress that air supply, temperature, inorganic substances, reaction, drying, sunlight, plants, animals and other microorganisms all have important effects on rhizobia.

There has been some disagreement as to the relationship between rhizobia and free oxygen. Beijerinck (as reviewed by Fred et al (14)) spoke of the need for oxygen in cultures of rhizobia. However, he considered an oxygen tension somewhat lower than that of the atmosphere best for the growth of these bacteria. Schneider (47) stated that the nodule organism of sweet clover will grow under aerobic conditions, but he thought the organism a facultative anaerobe. In 1917 Wilson (58) made a similar report concerning the nodule bacteria of soybeans. Alicante (5) studied the viability of the nodule bacteria of legumes outside the plant. He found that oxygen seemed to be a limiting factor in the growth of rhizobia in solution since aeration by shaking the bottles increased growth of the bacteria.

Ockerblad (38) compared the survival of rhizobia of several cross inoculation groups on both solid and liquid media in containers with varying degrees of aeration. After 30 days he found that the percentage of viable bacteria was considerably lower in sealed containers irrespective of the medium used. He concluded that the chief factors responsible for a decrease in the number of viable bacteria are (a) anaerobic conditions, (b) accumulation of metabolic products of a toxic or

inhibitory nature, and (c) plasmolysis caused by concentration of soluble solids through evaporation. Similar results have been reported by Fred, Whiting and Hastings (18).

Gangulee (20) carried out an experiment in which he grew rhizobia in sterile soil in large test tubes. Half of these tubes were sealed with paraffin wax over alkaline pyrogallol and the rest were incubated with free access to the air. After thirty days the conditions were reversed and the experiment continued for another thirty days. The results of counts made at intervals during the experiment emphasize the beneficial effect of free access to air on reproduction of rhizobia.

There are three or four remarkable reports of survival of rhizobia in laboratory cultures. Edwards (11) reported that he had been able to secure excellent nodule production with cultures from red clover, white clover and alfalfa, respectively 16, 10 and 10 years old. These cultures had been kept at room temperature in Freudenreich flasks sealed with wax. The culture medium consisted of wood ash, maltose, agar and water. Stapp, as reviewed by Fred et al (14), also reported the 16 year survival of three cultures. These three were among 65 cultures sealed in glass tubes and stored at room temperature. They were in neutral carrot agar medium, a fact which Stapp considers significant. One of the most exhaustive studies of the viability of nodule bacteria has been carried out by Jones (28). He stored stock cultures of a large number of strains of rhizobia for 11 to 15 years in small Freudenreich flasks containing modified Ashby's agar. After 11 years the cultures were plated out on the same

modified Ashby's agar. Surprisingly, all 12 of the cultures showed typical rhizobium colonies. His results with alfalfa nodule bacteria which had been kept in a tightly corked two ounce Blake bottle are even more striking. After 15 years viable counts showed that the bottle contained 110,000,000 living bacteria.

Extensive studies have been made on the effect of temperature on the growth of rhizobia. Beijerinck, as reviewed by Fred et al (14), gives 0°-47° C. as the thermal growth range for these organisms. The results of much work since that time indicate that growth takes place between 0° and 50° C with an optimum between 20° and 28° C. Ockerblad (38), reported the thermal death point of rhizobia in liquid media to be between 59° and 61° C with 10 minute exposure.

Fred et al (14) list the substances necessary in quantity for the growth of rhizobia as water, carbon as complex compounds, phosphorus, sulphur, potassium, magnesium, calcium and perhaps iron. Other elements usually present in media in small amounts which may or may not be necessary are manganese, aluminum and boron. Pitz (41) noted the beneficial effect of calcium sulphate on the multiplication of Rh. trifolii both in synthetic and soil extract media. Truesdell (55) observed that a small amount of phosphorus increased the number of alfalfa nodule bacteria in sterilized soils.

Fulmer (19) reports that the addition of calcium carbonate or magnesium carbonate will greatly stimulate the growth of rhizobia from alfalfa or lupines in a sterilized acid soil. Thornton (53) and Alicante (5) also stress the im-

portance of calcium on the growth of legume bacteria outside the host plant.

In summary Fred et al (14) conclude that calcium, phosphorus and potassium salts favor the development of the nodule bacteria, especially in culture solutions and in certain soils. Phosphorus compounds appear to be especially active in this regard. The so-called stimulants - boron, manganese, etc. in the proper concentrations may exert a favorable effect, though evidence in their favor is not extensive.

Field observations have shown that leguminous plants bearing nodules are to be found in soils of widely different reactions. This tolerance on the part of the host plant suggests that the root nodule bacteria may be equally tolerant of change in reaction. Moore (36) reported that the nodule bacteria will stand any degree of acidity or alkalinity of the soil that will permit the growth of its particular host plant. Prucha (43) showed that the growth of the nodule bacteria of alfalfa on agar slants is adversely affected in the presence of small amounts of a mineral acid such as hydrochloric, while equivalent amounts of a base such as sodium hydroxide have no harmful effect.

Salter (46) reports that Ashby's mannitol medium having a neutral or slightly alkaline reaction is favorable for the reproduction of the clover bacteria. Fred and Loomis (16) confirmed these results. Fred and Davenport (15) made a study of the relation between hydrogen-ion concentrations and the growth of different microorganisms. They found that the alfalfa bacteria

are the most sensitive and the lupine bacteria the most resistant to acidity.

Virtanen, as reviewed by Fred et al (14), has carried out experiments on the resistance of legumes and legume bacteria to acidity. He found that in general the bacteria are more sensitive to acidity than are the plants. He gives the optimum pH for the bacteria as 6.5 to 7.5 and for the plants, about 6.5.

Thornton (53) in studying the factors affecting the longevity of rhizobia in Florida soils, found that they will survive for a considerable time in the absence of the host plant if the pH of the soil is above 6.0. Truog (56) and Wilson (60), also found that acidity was an important factor in the growth of rhizobia in soil. Wilson reports that the critical pH for alfalfa bacteria survival in soil is 6.4 and for white clover bacteria is 6.0. Fred and Davenport (15) have studied the effect of reaction on rhizobia grown in culture solutions. They found that the critical pH for alfalfa and sweet clover bacteria survival was 4.9, while for lupine bacteria it was 3.15. They also reported that legume bacteria were less adversely affected by alkaline conditions than they were by acid conditions. Bryan (9) obtained similar results by placing different species of rhizobia in soils of varying reaction. He made counts for a period of 75 days and found the critical pH for the survival of alfalfa bacteria to be 5.0, red clover 4.5 to 4.7, and soybean bacteria 3.5 to 3.9.

Like all non-spore forming bacteria, the rhizobia are sensitive to excessive drying. The effect of desiccation,

however, depends on several conditions - the nature of the substrate, reaction, temperature, etc. Giltner and Langworthy (21) carried out extensive studies on the longevity of rhizobia under various moisture conditions. They found that rhizobia survive a long time in air dry soil, probably because of the films of hygroscopic moisture which surround the particles. Protective colloids in the soil may also play a part in the survival phenomenon, as the bacteria appear to remain viable for a longer period in a clay loam than in a sand. The gum produced by the rhizobia themselves may also have a protective action. Vandecaveye (57) and Temple (52) have likewise reported upon the remarkable viability of rhizobia in air dried soil.

Sunlight, too, may also affect rhizobia. Albrecht (1) in studying this question allowed soil with nodule bacteria to dry in sunlight and then stored it for three years. At the end of this time he found enough viable bacteria to produce good infection. In a later paper, Albrecht (2) reported a further study of the effect of sunlight on nodule bacteria of red clover and soybeans. In this test, one half of a soil sample containing nodule bacteria was spread out in a thin layer about quarter of an inch deep, and was exposed to direct sunlight until dry. After drying the soil was exposed for two months on a greenhouse bench, and then it was stored in a closed container. The other portion was dried in the dark before being similarly exposed and stored. Tests of the sun dried soil and of that dried in the dark showed survival of nodule bacteria in both. Albrecht's conclusion, that there is no evidence that the nodule bacteria are especially

sensitive to sunlight, seems justified. A more severe test was reported by Albrecht and Turk (4) in 1930. Knowing that it is the ultra-violet fraction of sunlight which is fatal to most bacteria, Albrecht and Turk exposed soil containing the nodule bacteria to strong radiation with ultra-violet light from a mercury arc lamp. They found no detrimental effect. The absorptive capacity of the soil apparently prevents penetration of the harmful rays.

According to Erdman and Wilkins (11), exposure of either inoculated soybeans or inoculated soil to sunlight does not seriously injure the "inoculating efficiency" of the nodule bacteria.

A paper by Nobles (37) reported the only evidence of the harmful effect of sunlight on rhizobia. He claimed that the nodule bacteria are quite sensitive to direct sunlight, although the compactness of the soil offers some protection. For example, he found that 80 percent of the bacteria in a sandy soil were destroyed by fifteen minutes' exposure to the sun, while about 54 percent were destroyed in a more compact soil.

Another factor which appears to influence the growth of rhizobia in soil is the symbiotic or antagonistic effect of other micro-organisms. The nodule bacteria have unquestionably existed in the soil and in the tissues of the leguminosae for ages. The reason that they are not more abundant and more widely distributed may be that the antagonism of other soil microorganisms holds them in check. As early as 1899, Nobbe and Hiltner (as reviewed by Fred et al (14)) suggested that the competition between

rhizobia and the rest of the soil flora results in harm to the rhizobia. They implicated both bacteria and fungi in the opposition. Sackett (45) 1906, in a laboratory study of associated growth of B. ramosus and rhizobia, observed that multiplication of the latter is greatly inhibited. Simon (as reviewed by Fred et al (14)), reported that soil flora is definitely antagonistic to root nodule bacteria. He also found that fungi are particularly harmful. Duggar and Prucha (10) obtained better growth of rhizobia in sterilized than in unsterilized soil and argued that the general soil flora have an antagonistic action towards rhizobia.

At Wisconsin Koniski (30) obtained some interesting data on the relation of certain soil bacteria to rhizobia. He isolated several unidentified organisms from the soil and tested them out with rhizobia in a liquid medium, with and without calcium carbonate. He also tested them in soil. He found inherent differences in the degree of antagonism exhibited by the cultures and also differences according to the conditions of the experiment. In general, antagonistic effects of other microorganisms towards rhizobia were most severe in liquid media without calcium carbonate and least severe in soil. Certain known cultures of soil bacteria were tested and were also found most injurious in liquid media. For example, B. subtilis inhibits Rh. meloliti, Rh. leguminosarum, and Rh. japonicum in liquid cultures but not in soil. The other organisms tested, B. mycoides, B. megatherium, B. prodigiosus, B. fluorescens and B. aerogenes are also harmless in soil, but have a depressive effect on rhizobia in liquid cultures.

There are also reports of beneficial effects of certain organisms upon rhizobia. Bottomley (8) reported enhanced nitrogen fixation by mixed cultures of nodule bacteria and Azotobacter. Manns and Goheen (34) and Fellers (13) referred to a beneficial effect from the association of Azotobacter and rhizobia upon colony formation in petri plates. Lohnis and Hansen (31) noted the stimulating effect of B. radiobacter upon colony formation of the cowpea-soybean cross inoculation group.

Another factor in the biological environment of rhizobia in soil is the activity of protozoa. In a study of the food requirements of soil amoebae, Severtzova (49) has shown that on artificial media these organisms destroy large numbers of bacteria. Of 26 species studies, 21 were eaten readily and 5 very little or not at all. The organisms from the nodules of leguminous plants are listed among the edible species. This indicates that amoebae may play an important role in the destruction of rhizobia in the soil. Somewhat contradictory results were reported by Hopkins (as reviewed by Fred et al (14)). He found that of 41 strains of rhizobia tested upon nitrogen free agar containing 1:100,000 crystal violet, only 12 strains were fed upon by amoebae. The availability of legume bacteria to amoebae appears to depend upon the rate of growth of the bacteria and the type of gum produced in the medium by rhizobia. Strains producing heavy gum are not attacked by amoebae. Losina-Losinsky and Martinov (32) reported an investigation of the diffusion of pure

cultures of Rh. trifolii, an amoeba and a ciliate planted together in the centre of a petri dish containing sterile soil. All three types of micro-organisms multiplied rapidly and spread outward at a rate seemingly controlled by the moisture content and mechanical structure of the soil. The rhizobia always preceded the protozoa and served as food for them.

Hino (24) presented another side to the picture. He found soil microorganisms roughly of two types: those promoting the growth of soil protozoa and those inhibiting their growth. Strangely enough, he lists the rhizobia as having an inhibitory effect on the growth of soil protozoa.

Considerable work has been done concerning the effect of steam sterilization on the chemical and physical properties of soil. More attention has been paid to the effect of steam sterilization on the chemical changes in the inoculant bases used than to its effect on the physical changes, although it is realized that both may be important.

The effect of steam sterilization on the properties of soil and peat has been reviewed by Simpson (50) in his study of the factors affecting steam sterilization of some Alberta soils. A brief review is repeated here since these changes are of importance in the work reported herein.

Schreiner and Lathrop (48) have obtained some interesting results from their investigation of the chemistry of steam heated soils. They steamed two silt loams at 30 pounds pressure

(135°C) for three hours. On extracting the soil with 2 percent sodium hydroxide they found a large increase in soluble solids. Analysis of the extract revealed an increase in the ammonia and the amino acid content and, in addition, the presence of new organic compounds such as arginine, cytosine, guanine, xanthine, and hypoxanthine, which were absent before heating. They concluded that these compounds resulted from the heat destruction of the nucleic acid and the protein material of the soil.

Russel and Hutchinson (44) observed that steaming a soil at atmospheric pressure resulted in an increase in the unstable nitrogen compounds and therefore concluded that something more than a simple liberation of ammonia was involved. The increase in ammonia obtained was about five parts per million. A smaller increase in nitrate was recorded.

Potter and Snyder (42) applied dry heat and steam heat under pressure to three different silt loams and a peat. Their results are consistent with those of other workers in that with the application of dry heat the ammonia and amino acid contents were increased, and in that the nitrates were slightly increased at 100°C, but were lost by heating soils at 200°C. This also held true for the peat except that the relative increases were smaller, probably owing to the loss of ammonia to the atmosphere.

Kelly and Thompson (29) reported that steam heat applied to soils at 2 atmospheres pressure produced a chemical change similar to that produced by dry heat at 150°C. At 200°C all of the lateritic soils used in the investigation gave increases in ammonia. A few soils were found to have a decreased content of

amide nitrogen while others were found to have an increased content after heating.

Simpson (50) carried out experiments on the factors affecting the steam sterilization of three typical Alberta soils and a peat. The three soils used included a brown virgin loam from Vauxhall, a black loam from a cultivated field near Edmonton, and a gray wooded loam from near Breton. Besides studying the time required to steam sterilize these soils, he studied the effect of sterilization upon their ammonia and nitrate nitrogen contents. He found that in all cases steam sterilization increased the ammonia content as determined by the McLean and Robinson (33) method. This increase was more marked when soils were sterilized in the moist condition than when they were sterilized air dry, despite the more severe treatment required to sterilize air dry samples. He found a greater increase in the ammonia content of peat on sterilization than was the case with the mineral soils. Steaming under pressure did not appear to produce significant changes in the nitrate content of the samples, but tended to reduce the amounts present.

OUTLINE OF INVESTIGATION.

The work reported in this thesis is concerned with factors influencing the growth of rhizobia in humus and soil cultures. The object of this study was to determine the effect of moisture, aeration and composition of the growth medium on the growth curve of rhizobia at room temperature. Four different inoculant bases or media were used for culturing the rhizobia. These materials were:

1. Winterburn peat - this is a high lime peat obtained in a bog at Winterburn, which is several miles west of Edmonton. The peat used was fairly well decomposed material obtained at a depth of approximately four feet.
2. Peat-black soil mixture - this base was prepared by mixing equal portions by weight of the base described above with a black mineral soil from the University plots just south of Edmonton.
3. Peat-carbon black mixture - this inoculant base consisted of a fine, well-decomposed peat obtained near Edmonton. The peat was mixed with a small amount of carbon black. This material was obtained through the courtesy of Mr. J. A. Robertson, of Edmonton, who had used it as an inoculant base in preparing commercial cultures of rhizobia.
4. Edmonton loam - this is a black zone soil from the University plots just south of the City of Edmonton. This soil comprised one-half of inoculant base number two.

Using the four bases listed above, incubation experiments were conducted with Rh. trifolii and Rh. leguminosarum.

Containers holding the various bases were inoculated with these two organisms and were incubated under a variety of conditions. Plate counts of the bacteria were made at intervals during the incubation period - usually at 10, 40, 70 and 140 days after inoculation.

The plan of the experiments is shown in Table I.

As indicated in Table I, incubations were carried out in paper cartons for two of the experiments, IV and VII. In the other experiments incubations were carried out in erlenmeyer flasks. Where cartons were used they were waxed over with paraffin at the beginning of the incubation period to prevent too rapid a loss of moisture. When incubation was carried out in erlenmeyer flasks, some of the flasks were tightly stoppered, whereas others were stoppered loosely so that a free exchange of air was possible. To stopper flasks tightly, rubber stoppers waxed over with paraffin were used. In stoppering flasks loosely, on the other hand, the practice was to use a rubber stopper one size smaller than would normally have been used, and to wrap a little absorbent cotton around it. This allowed for a free exchange of air in the erlenmeyer flask, but the drying out was less rapid than would have been the case had an ordinary cotton stopper been used.

In those cases where the inoculant base was supplemented by the addition of nutrients, the following mixture was used:

Sucrose	..	100	gms.
K_2HPO_4	..	10	"
NaCl	..	2	"
$MgSO_4$	..	4	"
$CaCO_3$	..	60	"

Table I. Plan of the experiments for studying the growth curves of *Rh. leguminosarum* (experiments I-IV) and *Rh. trifoli* (experiments V-VII) in humus and soil cultures.

	Bases Used	Stopperings	Treatments		Incubation Periods
Expt. I	Peat Peat-Black Soil Peat-Carbon mixture Black Soil	Tightly stoppered Cotton stoppered	Sterile and unsterile	No nutrients added	0 days 10 " 38 " 72 " 148 "
Expt. II	Same as above	All cotton stoppered	All sterile	Nutrients added to half of flasks	0 days 14 " 42 " 90 " 141 "
Expt. III	Peat-Carbon mixture Black Soil	All cotton stoppered	All unsterile	Nutrients added to half of flasks	0 days 12 " 25 " 70 " 112 "
Expt. IV	Peat Peat-Black Soil Peat-Carbon mixture Black Soil	Waxed paper cartons	Sterile and unsterile	No nutrients added	0 days 11 " 48 " 77 " 146 "

....continued next page.

Table I. cont'd.

	Bases Used	Stopperings	Treatments	Incubation Periods
Expt. V	Same as experiment I except *Rh.* *trifolii* used			0 days
				14 "
				38 "
				70 "
				142 "
Expt. VI	Same as experiment II except *Rh.* *trifolii* used			0 days
				20 "
				48 "
				89 "
				140 "
Expt. VII	Same as experiment IV except *Rh.* *trifolii* used			0 days
				8 "
				36 "
				68 "
				146 "

These salts and sucrose are used in the proportions given above in the media recommended by Fred et al (14) and by Fred and Waksman (17) for plating legume bacteria. It was added to the inoculant base at the rate of 1.4 percent by weight.

In a number of the experiments the growth curves of pure cultures of rhizobia were studied. In such cases the humus bases were sterilized in the autoclave at 15 pounds steam pressure (121°C). The peat, peat-black soil mixture and the peat-carbon mixture were held at this temperature for two and a half hours, while the black soil was heated in the autoclave at 121°C for 25 minutes. Simpson (50) found that this treatment gave complete sterilization of these bases in the amounts used here.

The inoculations were made using a suspension of the bacteria in a 0.75 percent sodium chloride solution. This suspension was added to the inoculant bases at the rate of 10 c.c. per 50 grams of soil. Counts of the number of legume bacteria in the suspension were made at the time of inoculation. From this the initial count of legume bacteria per gram of base could be calculated. All counts were made by the plate method using as the medium yeast-extract sucrose agar containing congo red, which is recommended by Fred et al (14), and by Fred and Waksman (17). The congo red aids in the detection of contaminants which might be present. This is possible since most of the contaminants absorb the dye, thus producing reddish colonies, whereas the colonies of the legume bacteria remain clear.

At the beginning of the incubation periods all bases were made up to 30 percent of their total water holding capacities. This 30 percent included the water carrying the suspension of nodule

bacteria. The tightly stoppered flasks remained at this water content throughout the incubation period. Weighings of the flasks at the beginning and at the end of the incubation period indicated that there was no moisture loss from these flasks. The loosely stoppered flasks and the paper cartons were also weighed before and after the incubation period, and their losses in weight recorded, thus enabling calculation of percentage moisture at the end of the period.

A secondary portion of this investigation consisted of the determination of the soluble or available nutrients in the various inoculant bases used. This was intended to correlate the effect of the various treatments with the chemical composition of the inoculant bases and to make a further correlation between chemical composition and the growth of the bacteria. Since this was a minor portion of the work, it was limited to a study of the availability of calcium, potassium, phosphorus, ammoniacal nitrogen, and nitrate nitrogen. The analyses were carried out using the method outlined by Peech and English (39).

In the analysis of the peat-carbon mixture it was found that there was more soluble organic matter than could be adsorbed by addition of activated charcoal to the extracting solution. Thus in analyzing this inoculant base a slight modification was used. After completing the extraction the filtered extract was heated with carbon black to remove the dissolved organic matter. Since this treatment volatilized the ammoniacal nitrogen and the nitrate nitrogen, it was impossible to determine the amounts of these ions in the peat-carbon base.

RESULTS.

The counts of bacteria and moisture percentages in the different inoculants receiving different treatments are given in Tables II to VIII. All counts are in millions per gram with the incubation times and treatments indicated in the table. Tables XI to XIV give the availability of the various elements in p.p.m. The treatment which the inoculant base received in each case is indicated in the table heading.

The counts of bacteria in the inoculant bases studied in this investigation are very closely correlated with the moisture content and aeration of the inoculants. This is particularly true of the counts of rhizobia, but is also very noticeable in the total counts of unsterilized inoculant bases.

Results in Table II show very definitely that in all four inoculant bases used, the numbers of rhizobia drop sharply when incubated in sealed flasks. This is particularly noticeable in the peat, peat-black soil and in the peat-carbon mixture. In these sterile bases the numbers of rhizobia rose for a time, being considerably higher when the second plate count was made than they had been at the time of inoculation.

In the third and subsequent counts in these three bases, the numbers of rhizobia had dropped below fifty thousand per gram.

In the fourth base, black soil, at 38 days the count of rhizobia was still higher than at the beginning of the incubation period. However, in this base numbers of rhizobia had

Table II. Effect of various treatments on plate counts of Rh. leguminosarum (B_3) and other microorganisms in four inoculants - peat, peat-black soil mixture, peat-carbon mixture and black soil (millions per gram).

Incubation time in days	Sterile Base. Peat. Stoppering						Unsterile Base. Stoppering					
	Rubber			Cotton			Rubber			Cotton		
	Nodule bacteria count	Total count	Per-cent H_2O	Nodule bacteria count	Total count	Per-cent H_2O	Nodule bacteria count	Total count	Per-cent H_2O	Nodule bacteria count	Total count	Per-cent H_2O
0	30.4	30.4	137.4	30.4	30.4	137.4	30.4	32.9	137.4	30.4	32.9	137.4
10	181.5	181.5	137.4	285.0	285.0	134.2	16.0	1011.0	137.4	15.0	819.0	135.0
38	0.0	0.0	137.4	129.0	129.0	127.4	0.0	224.0	137.4	12.0	1207.0	124.0
72	0.0	0.0	137.4	31.7	31.7	117.0	0.0	203.0	137.4	5.0	589.0	117.4
148	0.0	0.0	137.4	10.6	10.6	101.8	0.0	148.0	137.4	0.0	449.0	107.4
				Peat-Black Soil Mixture.								
0	30.4	30.4	81.0	30.4	30.4	81.0	30.4	31.9	81.0	30.4	31.9	81.0
10	292.0	292.0	81.0	341.0	341.0	78.8	11.5	275.0	81.0	3.3	416.5	78.3
38	0.2	0.2	81.0	190.0	190.0	72.4	0.0	205.0	81.0	7.0	261.0	71.0
72	0.0	0.0	81.0	238.0	238.0	57.6	4.0	116.0	81.0	5.5	195.0	63.2
148	0.0	0.0	81.0	50.0	50.0	42.0	0.0	158.0	81.0	0.0	131.0	50.2
				Peat-Carbon Mixture.								
0	30.4	30.4	69.2	30.4	30.4	69.2	30.4	100.2	69.2	30.4	100.2	69.2
10	490.0	490.0	69.2	570.0	570.0	67.2	20.0	396.5	69.2	8.8	784.0	67.4
38	0.0	0.0	69.2	160.0	160.0	60.8	0.0	101.0	69.2	9.0	921.2	62.6
72	0.0	0.0	69.2	376.0	376.0	50.8	0.0	193.0	69.2	0.8	617.0	53.2
148	0.0	0.0	69.2	62.0	62.0	27.8	0.0	507.0	69.2	0.0	401.2	34.2

.....continued next page.

Table II, cont'd.

Incubation time in days	Sterile Base. Stoppering						Unsterile Base. Stoppering					
	Rubber			Cotton			Rubber			Cotton		
	Nodule bacteria count	Total Count	Per-cent H_2O	Nodule bacteria count	Total count	Per-cent H_2O	Nodule bacteria count	Total count	Per-cent H_2O	Nodule bacteria count	Total count	Per-cent H_2O
Black Soil.												
0	30.4	30.4	29.2	30.4	30.4	29.2	30.4	30.9	29.2	30.4	30.9	29.2
10	265.0	265.0	29.2	303.0	303.0	27.6	58.0	58.0	29.2	50.0	55.0	28.0
38	60.0	60.0	29.2	258.0	258.0	20.2	98.0	183.0	29.2	287.0	454.0	22.0
72	0.0	0.0	29.2	142.0	142.0	16.6	3.3	10.6	29.2	1.6	6.4	15.0
148	0.0	0.0	29.2	0.0	0.0	5.0	2.9	11.2	29.2	0.0	59.0	6.6

dropped below fifty thousand per gram by the time the fourth count was made 72 days after the beginning of the incubation period.

In the unsterile, tightly stoppered flasks, results somewhat similar to those with sterile bases were obtained. In the peat, peat-black soil and peat-carbon mixture, when the third count was made no typical colonies of rhizobia appeared on the plates. There was a slight discrepancy in the peat-black soil mixture where a count of four million rhizobia per gram was obtained in the fourth count at 72 days, but this had again dropped below fifty thousand when the fifth count was made. An interesting fact brought out here is that, unlike the counts in the sterile containers, when the second count was made, ten days after incubation commenced, the count of rhizobia had dropped far below the initial numbers. This would seem to indicate that the suggestion made by Fellers (13), stating it is unnecessary to sterilize peat or soil when used as a base for culturing rhizobia, is incorrect. The presence of other soil organisms seems to exert a very strong antagonistic effect upon the growth of rhizobia. This is indicated by the fact that there was a high total count coupled with these low counts of rhizobia. The total count rose quickly from the commencement of incubation until the second count was made, and then fell quite sharply in the succeeding counts. In the peat-black soil mixture and in the peat-carbon mixture the plate counts indicated a rise in the total numbers of bacteria between the fourth and fifth counts. This may have been due to errors in the plate counts

or merely to variation in the different flasks. In the unsterile, tightly stoppered flasks containing black soil, the total count of bacteria did not rise as rapidly as in the other three inoculants. However, the count of rhizobia did rise quite markedly in this base, reaching 98 million per gram after 38 days incubation. The number of rhizobia as well as the total number of bacteria had dropped quite sharply in this base by the time the fourth and fifth counts were made.

In the cotton stoppered, sterile flasks the count had risen far above the number initially introduced when the second count was made. A marked drop was evident when the third count was made after 38 days incubation. A slight rise took place in the peat-carbon and peat-black soil mixtures between the third and fourth counts, while in the peat and in the black soil the numbers of rhizobia continued to drop. When the fifth count was made after 148 days incubation, no rhizobia were present in the black soil, while the peat-carbon mixture had the highest count, 62 millions per gram.

In the unsterile, cotton stoppered flasks as in the unsterile, tightly stoppered flasks, the numbers of rhizobia had dropped sharply in all bases except the black soil, when the second count was made. The number of rhizobia in the peat-black soil mixture rose slightly between the second and third counts but fell again thereafter. In all these bases no rhizobia were present when the final counts were made after 148 days incubation. Once again, as in the tightly stoppered flasks, counts of rhizobia and other bacteria in the black soil were higher after 10 days incubation than they had been initially. The number of

rhizobia had risen still higher after 38 days incubation. Numbers of rhizobia dropped quite sharply between the third and fourth counts and when the fifth count was made after 148 days incubation, none were found.

The total count in all four bases in unsterile, cotton stoppered flasks showed, in general, a sharp rise followed by a fall in numbers. In the peat, peat-carbon and black soil the largest number of bacteria were counted after 38 days incubation while in the peat-black soil mixture the largest number were counted after 10 days incubation.

Results of counts of rhizobia given in Table III show that where nutrients are added to the inoculant bases there is a marked increase in the numbers of nodule bacteria. The only exceptions to this are in the fourth counts of the numbers of rhizobia in the peat and the peat-black soil mixture. Here the number of rhizobia in the base to which no nutrients were added was higher than where nutrients were added. As in Table II, the results indicate the importance of moisture content on the numbers of rhizobia. The numbers rose for a time, but as the percentage of moisture in the base fell, the number of bacteria dropped quite markedly as well. The greatest numbers were present at the second count, with the exception of the black soil and the peat-carbon mixture having no nutrients added. Counts in these bases reached a maximum at the time the third count was made. The most marked increases in numbers of legume bacteria resulting from addition of nutrients were obtained in the peat-black soil mixture. In this base with nutrients added, the

Table III. Plate counts of Rh. leguminosarum (B_3) in the four inoculants incubated in erlenmeyer flasks, sterilized and stoppered with cotton (millions per gram).

Incubation time in days	Nutrients Added		No Nutrients Added	
	Nodule bacteria count	Percent H_2O	Nodule bacteria count	Percent H_2O
Peat.				
0	63.3	137.4	63.3	137.4
14	646.0	134.0	286.0	134.2
42	138.0	125.2	114.0	127.6
90	44.0	110.6	53.0	113.8
141	28.7	95.6	10.5	89.6
Peat-Black Soil Mixture.				
0	63.3	81.0	63.3	81.0
14	1794.0	77.4	526.0	78.6
42	1585.0	73.6	350.0	71.0
90	208.0	57.4	216.0	53.2
141	97.5	46.6	40.9	39.0
Peat-Carbon Mixture.				
0	63.3	69.2	63.3	69.2
14	1997.0	65.4	798.0	66.4
42	1617.5	59.2	918.0	56.6
90	1678.0	40.0	364.0	40.2
141	291.0	31.4	176.0	23.8
Black Soil.				
0	63.3	29.2	63.3	29.2
14	578.0	24.4	337.5	25.0
42	676.2	20.8	352.0	19.0
90	26.0	3.8	4.9	4.2
141	3.1	0.8	1.3	0.0

counts were nearly as high as in the peat-carbon mixture similarly treated. However, with no nutrients added, the counts of rhizobia in the peat-carbon mixture were considerably higher than in the peat-black soil mixture. Another interesting point in the data of this table is that the counts of rhizobia in the peat-carbon mixture treated with nutrients remained very high for 90 days. In all the other treatments, the numbers had dropped to a fraction of their highest count by this time.

Comparison of the counts in flasks to which no nutrients were added in Table III with those similarly treated in Table II, shows that these counts follow similar trends. In both cases the numbers increased for a time and then dropped sharply as the bases dried out.

Table IV gives the results from Experiment III. It was thought that it might be more practical, if possible, to use unsterilized inoculant bases. Therefore, in this experiment a study was made of the growth of rhizobia in unsterile inoculant bases with and without added nutrients.

In the peat-carbon mixture there seems to be no significant difference between counts of rhizobia in the base to which nutrients were added and that to which no nutrients were added. There was, however, a definite increase in the total number of bacteria in the base to which nutrients were added. This suggests that the competition effect of additional soil bacteria offset the beneficial effect derived by rhizobia from the nutrients added. In the black soil the addition of nutrients gave a very definite increase, in numbers of rhizobia as well as total count, over the

Table IV. Plate counts of Rh. leguminosarum (B_3) in two inoculants, unsterile and stoppered with cotton (millions per gram).

Incubation time in days	Nutrients Added			No Nutrients Added		
	Nodule bacteria count	Total count	Per cent H_2O	Nodule bacteria count	Total count	Per cent H_2O
			Peat-Carbon Mixture.			
0	101.2	181.0	69.2	101.2	181.0	69.2
12	15.0	1189.0	64.4	32.0	741.0	64.2
25	11.0	1021.0	62.0	9.0	425.0	54.8
70	4.0	541.0	41.4	5.0	406.0	48.4
112	0.1	512.0	32.4	0.4	308.0	35.2
			Black Soil.			
0	101.2	101.7	29.2	101.2	101.7	29.2
12	198.0	296.0	25.2	87.0	127.0	25.2
25	168.0	371.0	21.4	92.5	162.5	22.8
70	5.5	95.0	8.8	1.0	22.5	14.2
112	13.0	90.0	5.8	0.5	2.3	5.6

base to which no nutrients had been added. In the soil to which no nutrients were added, the numbers of rhizobia did not increase above the initial number added. Where nutrients were added, after twelve days incubation, the number of rhizobia had risen to approximately double the initial count. This is in marked contrast with the counts of rhizobia in the peat-carbon mixture, where after twelve days incubation the number of rhizobia present had dropped to a fraction of those originally present.

Table V contains the data of counts for bacteria in the inoculants stored in paper cartons. It includes counts for both sterile and unsterile inoculant bases. In the paper cartons

Table V. Plate counts of *Rh. leguminosarum* (B_3)in four inoculants, sterile and unsterile, incubated in paper cartons (millions per gram).

Incubation time in days	Sterile			Unsterile		
	Nodule bacteria count	Total count	Per cent H_2O	Nodule bacteria count	Total count	Per cent H_2O
Peat.						
0	35.8	35.8	137.4	35.8	38.3	137.4
11	134.0	-	134.2	8.0	908.0	130.4
48	46.0	-	128.6	13.0	945.0	105.0
77	72.0	-	114.0	1.0	754.0	102.6
146	4.3	-	86.6	0.0	541.0	63.2
Peat-Black Soil Mixture.						
0	35.8	35.8	81.0	35.8	37.3	81.0
11	210.0	-	77.8	14.0	277.5	78.6
48	107.5	-	69.0	6.0	271.0	62.6
77	103.0	-	59.4	3.0	229.0	63.4
146	0.0	-	30.6	0.0	130.0	30.2
Peat-Carbon Mixture.						
0	35.8	35.8	69.2	35.8	115.6	69.2
11	349.0	-	66.8	14.0	878.0	64.6
48	284.0	-	58.6	3.8	853.8	53.4
77	199.0	-	48.8	1.0	267.0	26.8
146	11.6	-	20.4	1.0	391.0	22.0
Black Soil.						
0	35.8	35.8	29.2	35.8	36.3	29.2
11	147.0	-	27.4	3.0	13.3	25.8
48	45.0	-	22.4	0.2	10.0	16.2
77	21.5	-	18.8	0.7	7.7	15.4
146	1.0	-	9.6	0.2	8.3	9.2

containing sterilized inoculant base, some recontamination did take place. However, this was infrequent and in such cases total counts were not recorded. In the sterilized bases the count of nodule bacteria rose quite high and remained at a fairly high level for the first 77 days incubation with the exception of the black soil. Here the count had fallen far below its maximum after 77 days incubation. Comparison of the data in this table with that in Tables II and III indicates that the numbers of rhizobia in sterile bases incubated in cartons do not rise as high as in similarly treated bases incubated in erlenmeyer flasks. The reason for the erlenmeyer flasks being better containers in which to culture rhizobia seems hard to determine. There are several factors which may have been contributory. Although total counts were not recorded, it was noticed that in many of the sterilized bases incubated in cartons appreciable mold growth had taken place on the surface of the base. The mold may have had an inhibitory effect on the growth of the rhizobia. The paper cartons, in spite of being waxed, usually retained a lower percentage of moisture at the end of the incubation period than did the bases stored in cotton stoppered flasks. This is another factor which could have contributed to the lower count of rhizobia in the bases stored in cartons. However, it is questionable if this could account for all the difference since in the early stages of incubation there was usually little difference between the moisture contents of the bases in cartons and those in flasks. A third possible factor is the difference in the initial number of bacteria introduced into the bases in the various experiments. In view of

the rapid multiplication of bacteria under favorable conditions, it seems questionable if this was of importance.

In the unsterilized bases incubated in paper cartons, a similar trend to that obtained in unsterilized bases incubated in erlenmeyer flasks is evident. Here the tendency was to get high total counts coupled with low counts of rhizobia. As in other counts of rhizobia in unsterilized inoculant bases, the numbers of rhizobia had dropped quite sharply by the time that the second plate counts were made. They continued to drop in subsequent plate counts. When the final counts were carried out on the peat and peat-black soil mixtures, no rhizobia were present, while in the peat-carbon black mixture and the black soil the numbers were 1 and 0.2 millions per gram respectively. The total count in the unsterilized bases rose quite high, except in the black soil, but dropped off gradually as the inoculant base dried out. In the black soil the total count was only 13.3 million after 11 days incubation and never rose above that figure in subsequent counts. In the sterilized base, on the other hand, after 11 days incubation there were 147 million rhizobia present per gram.

Table VI gives the data from Experiment V, an experiment which was identical with experiment I, except that red clover bacteria were used here instead of pea bacteria. It is therefore not surprising that the data in this table show similar trends to those found in the data of Table II. There are minor variations but these are not consistent enough to suggest any difference in the growth requirements of these two species

Table VI. Effect of various treatments on plate counts of *Rh. trifolii* (W239) and other microorganisms in peat, peat-black soil mixture, peat-carbon mixture and black soil (millions per gram).

	Sterile Base. Stoppering						Unsterile Base. Stoppering					
	Rubber			Cotton			Rubber			Cotton		
Incubation time in days	Nodule bacteria count	Total count	Per-cent H_2O	Nodule bacteria count	Total count	Per-cent H_2O	Nodule bacteria count	Total count	Per-cent H_2O	Nodule bacteria count	Total count	Per-cent H_2O
Peat.												
0	29.2	29.2	137.4	29.2	29.2	137.4	29.2	31.7	137.4	29.2	31.7	137.4
14	56.1	56.1	137.4	298.0	298.0	137.0	5.0	372.5	137.4	10.0	1270.0	137.0
38	0.4	0.4	137.4	124.0	124.0	134.0	5.0	180.0	137.4	3.0	1216.0	133.0
70	0.0	0.0	137.4	59.0	59.0	126.4	0.0	140.0	137.4	3.0	1167.0	124.6
142	0.0	0.0	137.4	5.0	5.0	103.0	0.0	50.5	137.4	0.0	712.0	106.2
Peat-Black Soil Mixture.												
0	29.2	29.2	81.0	29.2	29.2	81.0	29.2	30.7	81.0	29.2	30.7	81.0
14	456.5	456.5	81.0	320.0	320.0	80.0	1.3	72.3	81.0	1.3	80.4	80.0
38	46.0	46.0	81.0	187.0	187.0	78.0	1.0	1135.0	81.0	1.0	444.0	76.6
70	0.0	0.0	81.0	113.0	113.0	72.8	0.0	689.0	81.0	0.2	735.0	71.8
142	0.0	0.0	81.0	12.4	12.4	47.6	0.0	14.3	81.0	0.0	164.0	50.2
Peat-Carbon Mixture.												
0	29.2	29.2	69.2	29.2	29.2	69.2	29.2	109.0	69.2	29.2	109.0	69.2
14	286.0	286.0	69.2	846.0	846.0	67.0	4.0	237.0	69.2	19.0	835.0	67.4
38	0.0	0.0	69.2	628.0	628.0	60.0	2.0	260.0	69.2	2.5	835.0	59.0
70	0.0	0.0	69.2	776.0	776.0	52.6	0.0	125.0	69.2	1.1	752.0	54.2
142	0.0	0.0	69.2	47.0	47.0	30.0	0.0	116.5	69.2	0.0	330.0	32.6

.....continued next page.

Table VI. cont'd.

Incubation time in days	Sterile Base. Stoppering						Unsterile Base. Stoppering					
	Rubber			Cotton			Rubber			Cotton		
	Nodule bacteria count	Total count	Per-cent H_2O	Nodule bacteria count	Total count	Per-cent H_2O	Nodule bacteria count	Total count	Per-cent H_2O	Nodule bacteria count	Total count	Per-cent H_2O
						Black Soil.						
0	29.2	29.2	29.2	29.2	29.2	29.2	29.2	29.7	29.2	29.2	29.7	29.2
14	295.0	295.0	29.2	308.0	308.0	27.2	59.0	59.0	29.2	55.0	60.0	27.2
38	96.0	96.0	29.2	177.5	177.5	20.2	19.6	19.6	29.2	16.5	18.5	20.8
70	741.0	741.0	29.2	35.0	35.0	14.8	0.2	7.8	29.2	0.7	7.0	12.4
142	0.0	0.0	29.2	0.13	0.13	3.2	0.0	4.5	29.2	0.1	2.0	6.0

of bacteria. The differences can probably be accounted for on the basis of variability of the different flasks and variations in the plate counts.

In the tightly stoppered sterile flasks the number of rhizobia rose for the first few days and then dropped sharply. In each of the four inoculants the numbers of rhizobia had risen appreciably above the initial count after 14 days incubation. When the third count was made after 38 days incubation, the numbers of rhizobia had dropped significantly. When the fourth count was made after 70 days incubation, no rhizobia were found in the peat, peat-black soil mixture or peat-carbon mixture. In the black soil, for some reason, the numbers of rhizobia rose very rapidly between the third and fourth counts. This abnormally high count seems inexplicable on the basis of other results, particularly since there seemed to be no indication that air had entered the flask.

In the unsterile, but tightly stoppered, flasks, the results are again similar to those in Table II. The numbers of rhizobia dropped quite sharply from the beginning of the incubation period in the peat, peat-black soil mixture and in the peat-carbon mixture. There were no rhizobia present in these bases by the 70th day. In the black soil the number of rhizobia rose quite markedly at first. However, by the 70th day the number of rhizobia had dropped to 0.2 millions per gram.

In the sterile, cotton stoppered flasks the numbers of rhizobia rose quickly. The highest count was recorded at the second plate count after 14 days incubation. Thereafter the

numbers of rhizobia began to drop, the fall being most rapid in the black soil and least rapid in the peat-carbon black mixture where even at 142 days incubation there were 47 million rhizobia present per gram.

In the unsterile, cotton stoppered flasks, the numbers of rhizobia dropped considerably in all the bases, except the black soil, by the time the second count was made. In the black soil when the first count was made, the numbers of rhizobia had approximately doubled over those initially introduced into the base. When the third count was made after 38 days incubation, the numbers of rhizobia had again fallen below those initially introduced into the inoculant base. When the final count was made after 142 days, there were 0.1 million rhizobia per gram in the black soil and none were present in the other inoculant bases. The total counts in the peat, peat-black soil mixture and the peat-carbon mixture were quite high and remained at quite a high level until the fourth count, at which time the numbers in all these bases began to fall off. In the black soil no high total counts were obtained.

Table VII gives the results of counts from experiment VI, an experiment comparable to experiment II except that here red clover bacteria were used instead of pea bacteria. Comparison of the results in these two experiments indicates similar trends but with a tendency towards lower counts in experiment VI than in experiment II.

The number of rhizobia in the bases to which nutrients were added is, in every case but three (peat and peat-carbon mixture at 140 days and black soil at 20 days) definitely higher

Table VII. Plate counts of Rh. trifolii (W239) in four inoculants incubated in erlenmeyer flasks, sterilized and stoppered with cotton (millions per gram).

Peat.

Incubation time in days	Nutrients Added		No Nutrients Added	
	Nodule bacteria count	Percent H_2O	Nodule bacteria count	Percent H_2O
0	51.2	137.4	51.2	137.4
20	600.0	131.4	274.0	131.4
48	246.0	125.8	106.0	119.2
89	110.0	109.8	28.8	109.8
140	13.4	94.6	17.2	87.8
Peat-Black Soil Mixture.				
0	51.2	81.0	51.2	81.0
20	1287.0	75.2	493.0	75.6
48	731.0	60.4	253.0	64.4
89	180.0	58.6	114.0	54.6
140	22.9	42.4	12.9	28.0
Peat-Carbon Mixture.				
0	51.2	69.2	51.2	69.2
20	1132.0	64.0	673.0	63.8
48	1462.0	55.2	866.2	58.0
89	761.5	41.0	393.0	49.0
140	128.0	20.6	157.0	23.8
Black Soil.				
0	51.2	29.2	51.2	29.2
20	276.0	22.8	348.0	25.4
48	142.0	13.0	37.5	13.4
89	19.2	3.4	8.5	3.4
140	13.0	0.2	5.4	1.2

than the bases to which no nutrients were added. The highest counts were recorded at the second count in the peat, peat-black soil mixture and in the black soil, both where nutrients were added and where they were not added. In the peat-carbon mixture

the third count after 48 days showed the greatest number of rhizobia present. In general the highest counts were obtained in the peat-carbon mixture, the peat-black soil mixture being second in this regard, followed by peat and black soil in that order.

The data in Table VIII give the counts of red clover bacteria in inoculant bases stored in paper cartons. Here the numbers of rhizobia in the sterile base rose quite rapidly. The highest count was recorded at 8 days in the peat, peat-carbon mixture and in the peat-black soil mixture. In the black soil the highest count was recorded after 36 days incubation. Thereafter the numbers fell rapidly; after 146 days incubation the peat-carbon mixture, which had a count of 3.8 millions per gram, was the only base with more than 1 million rhizobia per gram. In the unsterile soil the counts of rhizobia dropped quite rapidly from the initial number of 33.3 million per gram in the peat, peat-black soil mixture and in the peat-carbon mixture. These bases contained correspondingly high total counts, which may, in part at least, account for the rapid drop in the numbers of rhizobia present. In the black soil the numbers of rhizobia rose considerably during the first 8 days of incubation, but by the time that the third count was made after 36 days incubation, the number of rhizobia had again fallen to a level below that initially present. When the final count was made at 146 days, there were no rhizobia present in either the black soil or in the peat-black soil mixture.

Table VIII. Plate counts of Rh. trifolii (W239) in four inoculants, sterile and unsterile, incubated in paper cartons (millions per gram).

Incubation time in days	Sterile			Unsterile		
	Nodule bacteria count	Total Count	Percent H_2O	Nodule bacteria count	Total count	Percent H_2O
			Peat.			
0	33.3	33.3	137.4	33.3	35.8	137.4
8	175.0	-	136.4	6.3	172.5	131.0
36	62.5	-	131.8	5.0	388.0	124.4
68	65.0	-	123.8	8.0	338.0	109.2
146	0.9	4.2	75.8	0.2	164.6	66.6
			Peat-Black Soil Mixture.			
0	33.3	33.3	81.0	33.3	34.8	81.0
8	191.5	-	80.0	3.9	87.4	79.4
36	105.0	-	69.6	9.0	183.0	72.2
68	10.7	-	68.8	1.2	53.2	64.0
146	0.1	13.6	42.0	0.0	9.6	28.4
			Peat-Carbon Mixture.			
0	33.3	33.3	69.2	33.3	113.1	69.2
8	569.0	-	67.6	2.2	72.6	68.8
36	233.0	-	64.0	7.0	177.0	67.0
68	52.0	-	57.8	1.0	255.0	64.2
146	3.8	9.1	33.8	0.3	116.4	21.4
			Black Soil.			
0	33.3	33.3	29.2	33.3	33.8	29.2
8	106.0	-	28.6	75.0	81.0	29.0
36	149.0	-	24.4	28.0	73.0	27.8
68	17.1	-	16.2	2.4	27.0	24.6
146	0.7	3.4	11.8	0.0	5.4	17.8

The results of plate counts of rhizobia given in these tables are in agreement with those of some workers and at variance with those of others. Ockerblad (38), Fred, Whiting and Hastings (18), and Gangulee (20) found aeration to be an important factor in the growth of rhizobia, and results reported here support these findings, whereas Edwards (11) and Jones (28) report

satisfactory growth of various species of these bacteria in sealed containers. Duggar and Prucha (10) report better growth of rhizobia in sterile than in unsterile soil. Sackett (45) and Nobbe and Hiltner (reviewed by Fred et al (14)) report that the general soil flora is antagonistic to rhizobia. Experiments reported here show clearly that under conditions of these experiments sterile bases were much superior to unsterilized bases for culturing rhizobia. This result does not conflict with reports made by Bottomley (8), Manns and Goheen (34), Fellers (13) and Lohnis and Hansen (31), since no attempt was made to study growth of rhizobia with pure cultures of specific soil organisms as did these workers. Truesdell (55) reports the beneficial effect of phosphorus on the growth of alfalfa bacteria in sterile soil; Temple (52) found that the addition of various energy sources increased the growth of rhizobia in a similar medium, while the beneficial effect of calcium on the growth of rhizobia outside the host plant has been noted by many workers, including Pitz (41), Fulmer (19), Thornton (53) and Alicante (5). There is general agreement between these findings and results reported here.

It is realized that it would have been desirable to supplement the plate counts of rhizobia in the inoculants with cultural experiments. However, time and facilities did not permit the carrying out of such work. Nevertheless a table has been set up to indicate the length of time for which the various inoculant bases remained effective. This is based on a statement made by Fellers (13). He found that cultures which contained more

than one million rhizobia per gram usually were effective in producing nodules on legume plants when applied to the seed before planting. Such a criterion of effectiveness of cultures is at best merely a guide.

In the analysis of the various inoculant bases for available nutrients, there were, for the most part, few clear cut differences between the different treatments.

Table XI gives the data for the unsterilized and uninoculated bases to which nutrients were added and for similarly treated bases to which no nutrients were added. The results for the calcium here are quite typical of those found for all the treatments. The amount of available calcium appears quite high in all the bases, as determined by this method, but shows little variation. Some differences are recorded in the amounts of available potassium. The data show an increase in available potassium in every case where nutrients are added to the inoculant base. The content of available potassium shows a particularly marked increase in the peat on the addition of nutrients. The content of available potassium is quite markedly higher in the peat-carbon mixture and in the black soil than in the other two inoculant bases. In the content of available phosphorus, as in the case of available potassium, there are some variations between the inoculant bases. The available phosphorus in the peat and in the peat-black soil mixture is quite low, while in the other two bases it is fairly high. After addition of nutrients to the soil, increases of available phosphorus are shown in all the bases except the black soil where for some reason there was no increase shown. The content of nitrate nitrogen is quite low in the peat

Table IX. Incubation periods between which *Rh. leguminosarum* fell from over one million per gram to less than one million per gram.

Treatment	Peat	Peat-Black Soil Mixture	Peat-Carbon Mixture	Black Soil
Sterilized flasks, no nutrients added, tightly stoppered.	10-38	10-38	10-38	38-72
Sterilized flasks, no nutrients added, cotton stoppered.	more than 148	more than 148	more than 148	72-148
Unsterilized flasks, no nutrients added, tightly stoppered.	10-38	10-38	10-38	more than 148
Unsterilized flasks, no nutrients added, cotton stoppered.	72-148	72-148	38-72	72-148
Sterilized flasks, nutrients added, cotton stoppered.	more than 141	more than 141	more than 141	more than 141
Sterilized flasks, no nutrients added, cotton stoppered.	more than 141	more than 141	more than 141	more than 141
Unsterilized flasks, nutrients added, cotton stoppered.	-	-	70-112	more than 112
Unsterilized flasks, no nutrients added, cotton stoppered.	-	-	70-112	70-112
Sterilized cartons, no nutrients added.	more than 146	77-146	more than 146	146*
Unsterilized cartons, no nutrients added.	77-146	77-146	146*	11-48

- not determined.

* count of 1 million per gram at 146 days.

Table X. Incubation periods between which Rh. trifolii fell from over one million per gram to less than one million per gram.

Treatment	Peat	Peat-Black Soil Mixture	Peat-Carbon Mixture	Black Soil
Sterilized flasks, no nutrients added, tightly stoppered.	14-38	38-70	14-38	70-142
Sterilized flasks, no nutrients added, cotton stoppered.	more than 142	more than 142	more than 142	70-142
Unsterilized flasks, no nutrients added, tightly stoppered.	38-70	38*	38-70	38-70
Unsterilized flasks, no nutrients added, cotton stoppered.	70-142	38*	70-142	38-70
Sterilized flasks, nutrients added, cotton stoppered.	more than 140	more than 140	more than 140	more than 140
Sterilized flasks, no nutrients added, cotton stoppered.	more than 140	more than 140	more than 140	more than 140
Sterilized cartons, no nutrients added.	68-146	68-146	more than 146	68-146
Unsterilized cartons, no nutrients added.	68-146	68-146	68*	68-146

* one million bacteria per gram.

and peat-black soil mixtures and is appreciably higher in the black soil. The content of ammoniacal nitrogen is fairly high in the peat and peat-black soil mixture and is generally lower in the black soil. The pH of the peat and peat-black soil mixture here as in the other tables is slightly on the acid side, while the peat-carbon mixture and the black soil are both approximately neutral. The addition of the nutrient mixture, which contained calcium carbonate, resulted in a slight rise in pH in every case.

Table XI. Reaction and availability of nutrient elements in unsterilized and unincubated inoculant bases (availability in p.p.m.)

Element	Peat	Peat-Black Soil Mixture	Peat-Carbon Mixture	Black Soil
	No nutrients added.			
Ca	3000	3000	3000	3000
K	65	115	200	150
P	5	20	40	40
NO_3-N	20	40	-	60
NH_3-N	100	100	-	25
pH	5.9	6.0	7.0	7.0
	Nutrients added:	(Ca-1400 p.p.m. (K - 300 p.p.m. (P - 125 p.p.m.		
Ca	2500	2500	3000	2000
K	250	250	300	300
P	40	40	80	40
NO_3-N	10	30	-	50
NH_3-N	65	50	-	50
pH	6.0	6.2	7.1	7.1

- these determinations not made.

Table XII gives the data for available nutrients in bases which had received similar treatments to those analyzed in Table XI, except that here they were sterilized. As before, the amount of available calcium is generally high, showing little variation. The content of available potassium is again quite low in the peat but shows a marked increase where the nutrients, which included potassium, were added. In the other three bases the amounts of available potassium were generally higher than in the peat and were not increased by the addition of nutrients. It is possible that the sterilization treatment may have been responsible for this. The quantities of available phosphorus are

Table XII. Reaction and availability of nutrient elements in sterilized, unincubated inoculant bases (availability in p.p.m.)

Element	Peat	Peat-Black-Soil Mixture	Peat-Carbon Mixture	Black Soil
		No nutrients added.		
Ca	3000	3000	3000	2000
K	25	145	300	275
P	10	20	80	40
NO_3-N	15	40	-	50
NH_3-N	200	200	-	50
pH	6.0	6.2	6.9	6.9
	Nutrients added:	(Ca-1400 p.p.m. (K - 300 p.p.m. (P - 125 p.p.m.		
Ca	2000	3000	3000	2000
K	150	150	300	140
P	10	20	100	30
NO_3-N	20	40	-	50
NH_3-N	200	200	-	75
pH	6.0	6.3	7.1	7.2

- these determinations not made.

quite low in the peat and peat-black soil mixture, but are appreciably higher in the black soil and in the peat-carbon mixture. No significant changes are apparent in the bases to which nutrients were added over that to which no nutrients were added. The amounts of nitrate nitrogen are again, as in Table XI, quite low in the peat and peat-black soil mixture, and are somewhat higher in the black soil, being more or less unaffected by the addition of nutrients. This is not surprising since the nutrient mixture contained no nitrogen. The content of ammoniacal nitrogen was very markedly increased by sterilization, particularly in the peat and the peat-black soil mixture and to a lesser extent

in the black soil. This is in keeping with the findings of Simpson (50). The reaction of these four bases appears to be unchanged by the sterilization, since the differences between the pH's recorded in Table XII and those recorded in Table XI are well within error of successive determinations.

Table XIII gives the data for available nutrients in inoculant bases which were sterilized and then incubated. The amounts of available calcium again show no variation. The figures for available potassium are quite high but show no consistent variation with addition of nutrients. The content of available phosphorus is low in the bases to which no nutrients were added, being slightly increased by the addition of the nutrient salts as shown by the tests. The amounts of nitrate nitrogen are quite low except in the black soil and peat-black soil mixture to which no nutrients were added. Here quite an appreciable amount was found. As in the experiments recorded in Table XII, the amounts of ammoniacal nitrogen are increased in the peat and peat-carbon mixture by sterilization. The content of ammonia remained quite low in the black soil despite the sterilization. The pH's of the bases seem to be unaffected by the sterilization and incubation, but are frequently slightly increased by the addition of the nutrient mixture.

Table XIV gives the data for the inoculant bases which were unsterilized and incubated. Here also little can be concluded from the results of the calcium analysis except that this element is present in quite large quantities.

Table XIII. Reaction and availability of nutrient elements in sterilized and incubated inoculant bases (availability in p.p.m.)

Element	Peat	Peat-Black Soil Mixture	Peat-Carbon Mixture	Black Soil
No nutrients added - incubated 158 days.				
Ca	3000	3000	3000	2000
K	125	250	300	250
P	5	20	40	30
NO_3-N	Tr.	50	-	60
NH_3-N	375	315	-	50
pH	5.9	6.1	7.1	6.9
Nutrients added: (Ca-1400 p.p.m.) (K - 300 p.p.m.) (P - 125 p.p.m.) - incubated 134 days.				
Ca	3000	3000	3000	3000
K	200	200	300	300
P	20	40	80	40
NO_3-N	15	20	-	Tr.
NH_3-N	250	180	-	25
pH	6.0	6.3	7.0	7.1

- these determinations not made.

The results for available potassium are again quite high, showing little significant difference between bases to which nutrients were added and those to which no nutrients were added. The amounts of phosphorus are low in the peat and peat-carbon mixture and higher in the other two bases, but are unchanged by the addition of nutrients. In the bases to which no nutrients were added, quite interesting results were obtained in the ammonia and nitrate nitrogen determinations. The latter were high while the former were quite low, indicating that nitrification had taken place. In the black soil where nutrients were added, these results were not obtained; in this case both the nitrate nitrogen and the ammoniacal nitrogen contents were low. In this table the results for reaction of the inoculants showed some variation from

Table XIV. Reaction and availability of nutrient elements in unsterilized and incubated inoculant bases (availability in p.p.m.)

Element	Peat	Peat-Black Soil Mixture	Peat-Carbon Mixture	Black Soil
No nutrients added - incubated for 158 days.				
Ca	3000	3000	3000	3000
K	125	250	300	250
P	10	20	80	30
NO_3-N	250	200	-	80
NH_3-N	15	15	-	15
pH	5.5	5.8	7.3	6.8
Nutrients added: (Ca-1400 p.p.m.) (K - 300 p.p.m.) (P - 125 p.p.m.) incubated for 103 days.				
Ca	-	-	3000	2000
K	-	-	250	300
P	-	-	80	40
NO_3-N	-	-	-	15
NH_3-N	-	-	-	15
pH	-	-	7.1	7.0

- these determinations not made.

what might have been predicted. The pH values of the peat and of the peat-black soil mixture are considerably lower than in the other tables. The reactions of the other two bases are quite in line with what might have been expected.

In conclusion it might be said that the method of analysis used did not appear to be very sensitive. There are practically no consistent significant differences in availability of elements in the bases receiving different treatments. Although quite large amounts of potassium, phosphorus and calcium were added in the nutrient mixture, these additions are not always reflected in the results of the analysis. This is hard to explain,

particularly since the growth of bacteria, both legume and general soil flora, was significantly increased by the addition of these nutrients. There is, however, one apparently significant and consistent difference shown by these data. The amounts of ammoniacal nitrogen are greatly increased in the peat and the peat-black soil mixture by sterilization.

It is felt that this study of the factors which affect growth of rhizobia in humus and soil cultures should be continued. Cultural experiments in which inoculants are applied to legume seeds to be grown under bacteriologically controlled conditions should be included. Such work should compare commercial inoculants with ones such as those reported here. These comparisons should involve plate counts of rhizobia in cultures and a determination of their effectiveness in producing nodulation on leguminous plants.

A need for two other types of study is indicated also. It would seem desirable to make a more comprehensive study of the effect of addition of nutrient elements such as phosphorus, calcium and potassium, various energy sources and vitamins to the inoculant bases.

Results indicate the desirability of using pure culture commercial inoculants stored in a type of container allowing aeration. A study of containers is therefore needed in order to develop the most suitable type for commercial inoculants.

SUMMARY.

1. The work reported here is a study of the factors influencing the growth of rhizobia in humus and soil cultures. The object of this study was to determine the effect of moisture, aeration, and composition of the growth medium on the growth curve of rhizobia at room temperature. Four inoculant bases were used in this work - peat, a mixture of peat with black soil, a mixture of peat with carbon black and a black soil.

2. The growth of rhizobia is affected by the percentage moisture in the base. At 30 percent of the water holding capacity of the base very rapid growth takes place and high counts of rhizobia are obtained for several weeks. As the base dries out, the numbers decrease sharply.

3. The presence of other soil microorganisms has an inhibitory effect on the growth of rhizobia. Higher counts of rhizobia were obtained in the sterile than in the unsterile bases. The difference between numbers of rhizobia in the sterile and unsterile bases was less noticeable in the black soil than in the other bases.

4. When no aeration was allowed, the rhizobia, although they increased for a time in the sterile base, soon died out completely.

5. Addition of a nutrient mixture containing sucrose, calcium carbonate, di-potassium hydrogen phosphate, sodium chloride, and magnesium sulphate increased the counts of rhizobia in the sterile base. In unsterile base the beneficial effect of nutrients was

offset by the inhibitory effect of other microorganisms.

6. The counts of rhizobia in sterile bases were lower when stored in paper cartons than when incubated in erlenmeyer flasks. This may have been due to recontamination of the cartons.

7. The growth curves of Rh. leguminosarum (pea bacteria) and of Rh. trifolii (red clover bacteria) were similar.

8. When sterilized, the peat-carbon mixture was the best of the four inoculant bases studied for the culture of rhizobia. In the unsterile condition the highest counts of rhizobia were obtained in the black soil.

9. A summary of the incubation periods during which the numbers of rhizobia fell from above one million per gram to below that figure are given in Tables IX and X.

10. There is a positive correlation between the amounts of phosphorus, potassium, and calcium and the numbers of rhizobia the base will support. Addition of these elements increased the growth of rhizobia.

11. The amount of ammoniacal nitrogen was increased by sterilization of the high humus bases.

12. Nitrification took place during incubation of some of the unsterilized bases.

13. The method of analysis used to determine easily soluble nutrients did not seem to reflect addition of calcium as calcium carbonate. The tests reflected the additions of phosphorus and potassium but not in the amounts added.

14. Sterilization of these bases did not affect their reaction. Addition of calcium carbonate usually resulted in a slight increase in pH.

ACKNOWLEDGMENTS.

The writer wishes to express his thanks and appreciation to Dr. J. D. Newton, Department of Soils, under whose direction this work was carried out, for his helpful advice and criticism during the course of this work and in the preparation of the manuscript; to Dr. F. A. Wyatt, Head of the Department of Soils, for placing the facilities of the department at his disposal. Acknowledgment is also due to Dr. C. F. Bentley for his advice and criticism in the preparation of this manuscript; to F. J. Simpson for his help and advice regarding techniques and procedures, and to J. F. T. Spencer for his willing help at all times.

Funds supplied by the National Research Council, Ottawa, made this work possible.

REFERENCES

1. ALBRECHT, W. A. Studies on the longevity of B. radicicola in the soil. Missouri Agr. Expt. Sta., Bul. 189:54-56. 1921.

2. _____________. Viable legume bacteria in sun dried soil. Jour. Amer. Soc. Agron. 14:49-51. 1922.

3. _____________. "Dry inoculants" for alfalfa. Jour. Amer. Soc. Agron. 22:916-918. 1930.

4. ALBRECHT, W. A. and TURK, L. M. Legume bacteria with reference to light and longevity. Missouri Agr. Expt. Sta., Res. Bul. 132:19, pp. 1930.

5. ALICANTE, M. W. Viability of the nodule bacteria of legumes outside of the plant. Soil Sci. 21:93-114. 1926.

6. AMES, C. T. and CASANOVA, O. B. Inoculation. Missouri Agr. Expt. Sta., Bul. 264:38. 1928.

7. ARNY, A. C. and McGINNIS, F. W. Methods of applying inoculated soil to the seed of leguminous crops. Jour. Amer. Soc. Agron. 13:289-303. 1921.

8. BOTTOMLEY, W. B. The fixation of nitrogen by free-living soil bacteria. Brit. Assoc. Adv. Sci., Rpt. 80:581-582. 1910.

9. BRYAN, O. C. Effect of acid soils on nodule-forming bacteria. Soil Sci. 15:37-40. 1923.

10. DUGGAR, B. M. and PRUCHA, M. J. The behaviour of Pseudomonas radicicola in the soil. Science (N. S.) 35:229. 1912.

11. EDWARDS, S. F. A note on the longevity of some cultures of B. radicicola. Abs. Bact. 7:9. 1923.

12. ERDMAN, L. W. and WILKINS, F. S. Soybean inoculation studies. Iowa Agr. Expt. Sta., Res. Bul. 114:1-56. 1928.

13. FELLERS, C. R. Report on the examination of commercial cultures of legume infecting bacteria. Soil Sci. 6:53-67. 1918.

14. FRED et al. Root Nodule Bacteria and Leguminous Plants. Univ. of Wisc. Press., Madison, Wis. 1932.

15. FRED, E. B. and DAVENPORT, A. Influence of reaction on nitrogen assimilating bacteria. Jour. Agr. Res. 14:317-336. 1918.

16. FRED, E. B. and LOOMIS, N. E. Influence of hydrogen-ion concentration of the medium on the reproduction of alfalfa bacteria. Jour. Bact. 2:629-633. 1917.

17. FRED, E. B. and WAKSMAN, S. A. Laboratory Manual of General Microbiology. McGraw Hill Book Co. New York. 1928.

18. FRED, E. B., WHITING, A. L. and HASTINGS, E. G. Root Nodule Bacteria of Leguminosae. Wis. Agr. Expt. Sta., Res. Bul. 72:1-43. 1926.

19. FULMER, H. L. Influence of carbonates of magnesium and calcium on bacteria of certain Wisconsin soils. Jour. Agr. Research 12:463-504. 1918.

20. GANGULEE, N. Studies on the lucerne nodule organism (*B. radicicola*) under laboratory conditions. Ann. Applied Biol. 13:360-373. 1926.

21. GILTNER, W. and LANGWORTHY, H. V. Some factors influencing the longevity of soil micro-organisms subjected to desicsation, with special reference to soil solution. Jour. Agr. Res. 5:927-942. 1916.

22. HARTLEB, R. Methods of inoculating seeds with micro-organisms. U. S. Patent No. 674,765. May 21, 1901.

23. __________. Nutrient medium for producing cultures of bacteroids of micro-organisms. U. S. Patent No. 674,764. May 21, 1901.

24. HINO, J. Studies on soil protozoa. IV. Studies on the reproduction of protozoa as influenced by soil microbes inoculated in the culture. Internatl. Soc. Soil. Sci., Proc. 5:167-168. 1930.

25. HOFER, A. W. Numbers of rhizobium in humus cultures. Jour. of Bact. 31:95-96. 1931.

26. __________. Number of legume bacteria in commercial cultures as related to nodule formation. Jour. Amer. Soc. of Agron. 30:451-460. 1938.

27. Illinois Farmer's Institute. Hoard's Dairyman 45:766. 1913.

28. JONES, D. H. The viability of *Rhizobium legumenosum* Frank and *Rhizobium radicicolum* Beijerinck. Jour. Bact. 13:55-56. 1927.

29. KELLEY, W. P. and THOMPSON, A. R. The organic nitrogen of Hawaiian soils. II. Effects of heat on soil nitrogen. Jour. Amer. Chem. Soc. 36:434-438. 1914.

30. KONISHI, K. Effect of certain soil bacteria on the growth of the root nodule bacteria. Kyoto Imp. Univ. Col. Agr. Mem. 16. (Chemical Series 10):17 pp. 1931.

31. LOHNIS, F. and HANSEN, R. Nodule bacteria of leguminous plants. Jour. Agr. Res. 20:543-556. 1921.

32. LOSINA-LOSINSKY, L. and MARTINOV, P. F. A Method of studying the activity and rate of diffusion of protozoa and bacteria in the soil. Soil Sci. 29:349-362. 1930.

33. McLEAN, W., and ROBINSON, G. W. A new method for the determination of ammoniacal nitrogen in soils. Jour. Agr. Sci. 14:548-554. 1924.

34. MANNS, T. F. and GOHEEN, J. M. A preliminary report on muck humus as a fertilizer and carrier of beneficial soil bacteria. Del. Col. Agr. Expt. Sta., Bul. 115:1-14. 1916.

35. MOORE, G. T. Bacteria and the nitrogen problem. U. S. D. A. Yearbook 333-342. 1902.

36. __________. Soil inoculation for legumes; with reports upon the successful use of artificial cultures by practical farmers. U. S. Dept. Agr. Bur. Plant Indus., Bul. 71:1-72. 1905.

37. NOBLES, C. Spring inoculation of legumes. Mich. Agr. Expt. Sta., Quart. Bul. 1:100. 1919.

38. OCKERBLAD, F. O. Viability of *Pseudomonas radicicola* under aerobic and partial anaerobic conditions. Mich. State Bd. Agr. Ann. Rpt. 57:255-264. 1918.

39. PEECH, M. and ENGLISH, L. Rapid micro-chemical soil tests. Soil Sci. 57:167-195. 1944.

40. PERKINS, A. T. The effect of bacterial numbers on the nodulation of Virginia soybeans. Jour. Agr. Res. 30:95-96. 1925.

41. PITZ, W. The effect of elemental sulphur and of calcium sulphate on certain of the higher and lower forms of plant life. Jour. Agr. Res. 5:771-780. 1916.

42. POTTER, R. S. and SNYDER, R. S. The effect of heat on some nitrogenous constituents of soil. Soil Sci. 5:197-213. 1918.

43. PRUCHA, M. J. Physiological studies of *Bacillus radicicola* of Canada field pea. N. Y. (Cornell) Agr. Col., Mem. 5:1-83. 1915.

44. RUSSEL, E. J. and HUTCHINSON, H. B. The effect of partial sterilization of soil on the production of plant food. Jour. Agr. Sci. 3:111-114. 1909.

45. SACKETT, W. G. The association of *Pseudomonas radicicola* with *Bacillus ramosus*. Mich. Acad. Sci. 8:147-150. 1906.

46. SALTER, R. C. The behaviour of legume bacteria in acid and alkaline media. Iowa Acad. Sci., Proc. 23:309-313. 1916.

47. SCHNEIDER, A. Contribution to the biology of Rhizobia: 1. Rhizobium Mutabile in artificial culture media. Bot. Gaz. 34:109-113. 1902.

48. SCHREINER, O. and LATHROP, E. C. The chemistry of steam heated soils. U. S. D. A. Bur. Soils. Bull. 89. 1912. Also in Jour. Amer. Chem. Soc. 34:1242-59. 1912.

49. SEVERTZOVA, L. B. The food requirement of soil amoeba with reference to their interrelation with soil bacteria and soil fungi. Centbl. Bakt. 2 Abt. 73:162-179. 1928.

50. SIMPSON, F. J. Factors affecting steam sterilization of some Alberta soils. M.Sc. Thesis, Univ. of Alta. 1946.

51. SPAULDING, M. F. Comparative efficiency of humus and agar as carrier for legume bacteria of soybeans and peanuts. Proc. Soil Sci. Soc. of Amer. 5:259-261. 1940.

52. TEMPLE, J. C. Studies of Bacillus radicicola. I. Testing commercial cultures. II. Soil as a medium. Ga. Agr. Expt. Sta., Bul. 120:65-80. 1916.

53. THORNTON, G. D. Some factors affecting the longevity of rhizobium in Florida soils. Soil Sci. Soc. of Am., Proc. 8:238-239. 1943.

54. THORNTON, H. G. The influence of the number of nodule bacteria applied to the seed upon nodule formation in legumes. Jour. Agr. Sci. 19:373-381. 1929.

55. TRUESDELL, H. W. The effect of phosphorus on alfalfa and alfalfa bacteria. Soil Sci. 3:77-98. 1917.

56. TRUOG, E. Soil acidity: Its relation to the growth of plants. Soil Sci. 5:169-195. 1918.

57. VANDECAVEYE, S. C. Effect of moisture temperature and other climatic conditions on Rh. leguminosarum in the soil. Soil Sci. 23:355-362. 1927.

58. WILSON, J. K. Physiological studies of the Bacillus radicicola of the soybean (Soja Max Piper) and of the factors influencing nodule production. N. Y. (Cornell) Agr. Expt. Sta., Bul. 386:369-413. 1917.

59. ____________. Effect on nodulation of supplementing the legume bacteria of the soil with artificial cultures. Jour. Amer. Soc. Agron. 18:280-294. 1926.

60. ____________. Legume bacteria population of the soil. Jour. Amer. Soc. Agron. 18:911-919. 1926.

61. ____________. Acidity changes in stored legume seeds. Jour. Amer. Soc. Agron. 21:815-817. 1929.

B29753